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The Relation of Bacterium Vignae to the Tissues of Lima Bean

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The Relations of Bacterium Vignae to the Tissues of Lima Bean

W. S. Beach

Introduction

THE INTRICATE relations of parasitic bacteria to the cells of plants, the manner of migration through the tissues, and the changes produced by the parasitism are matters of great interest to the plant pathologist. The technical difficulties surrounding a study of such relations, however, in the past have prevented a full understanding of the many complicated features, or have left them in doubt. Recent advances in clarifying certain doubtful features have been made by Nixon's study (5, 6) of *Bacillus amylovorus* in apple twigs. Inasmuch as some outstanding features of the behavior of these bacteria within the host tissues were revealed, the great value of studying other types of bacterial diseases in similar respects and in a like manner became evident.

As an object worthy of investigation, a typical spot-forming organism was sought, and *Bacterium vignae* Gard. & Kendr. on lima bean was selected. This parasite produces upon the leaves of lima bean spots that are at first brown with distinct reddish-brown to purplish-brown borders. With age, the centers of the spots commonly become ashen and fall away. In size, these spots vary usually from 1 to 3 mm. in diameter (Fig. 1). On the pods, the disease begins as small brown spots surrounded by a water-soaked halo. Later these may become reddish-brown and depressed. In damp weather, it is not uncommon for a straw-colored exudate teeming with bacteria to appear upon pod or stem lesions. The differences in the character of the host, the parts parasitized, the type and extent of the lesions, and the ultimate condition of the invaded tissues made it appear probable that intimate histological study and comparison would reveal many new or diverse facts of interest. The manner of investigation would also permit examination of possible changes that the parasite undergoes during its course of life in the host tissue.

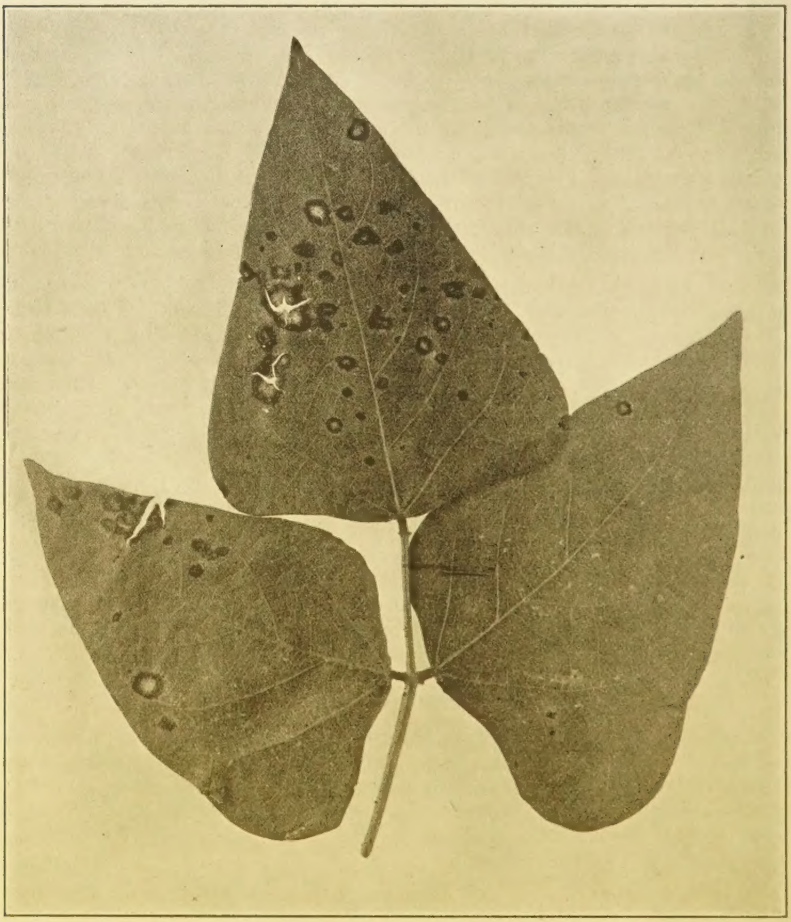
Literature Review

Bacterium vignae Gard. & Kendr. parasitizes the leaves, pods and stems of cowpea and lima bean and also a number of hosts closely allied to the latter. The various morphological and physiological characters distinguishing this parasite and the disease it causes have been reported by Tisdale and Williamson (9) for lima bean and by Gardner and Kendrick (3) for cowpea.

In lima bean, Tisdale and Williamson (9) report "that the organism enters through the stomata and works its way into the under-lying tissues between the cells. The middle lamella was destroyed and the cells were

crowded apart by the increasing numbers of bacteria. In later stages the walls may be torn or collapse and the bacteria invade the cell cavities. No vascular invasion was observed."

Gardner and Kendrick (3), in the case of cowpea, state, "Microscopic examination of the leaf lesions shows that invasion is intercellular and tends to be restricted to the mesophyll during the early stages. . . . In cowpea seedlings vascular infection has been frequently observed . . . Reddish-brown bundles were traced from the infected cotyledon or



epicotyl down into the hypocotyl and up through the epicotyl into the leaf veins. In water mounts, the bacteria were seen to ooze from the cut ends of these reddish-brown spiral vessels of the epicotyl, and in cross section these vessels appeared to contain bacteria. . . . When freshly cut edges of the leaf lesions were examined in water mount, the bacteria were seen to ooze from the cut ends of veins. In cross section the bacteria were noted in the intercellular spaces of the parenchyma of the veins but were not actually demonstrated within the spiral tubes at any distance from the leaf lesions. The organism very evidently shows a preference for the vein tissues and travels more rapidly in the veins than in the mesophyll."

Material and Methods

All the material prepared for histological study was obtained by inoculations made upon lima bean plants grown in the laboratory garden. The culture of bacteria used was isolated from naturally infected leaves collected at Bustleton, Pennsylvania. A photograph of the natural material is shown in Fig. 1. After the power of the culture to produce the typical disease spots had been proved, it was examined morphologically and grown upon the media necessary to determine its salient characteristics. The data obtained agreed with *Bacterium vignae* Gard. & Kendr.

Inoculations were made both by atomizing and with a fine needle. As a rule, immature leaves and pods were selected. Infection appeared to be more certain on immature tissues. The insertion of the needle was made at right angles to the epidermis and was deep enough in the case of pods to reach the interior of the fruit and some of the seeds. Thus, all series of tissues were reached.

Bacterium vignae on lima bean was found well adapted to a study of the various aspects of parasitism on account of the ease and certainty of infection. Nearly all inoculations with the needle were successful, while atomizing the leaves usually gave more or less numerous spots. The pod material was found to be much the better adapted for the detailed examination of the relation of the bacteria to the host cells, as the greater thickness of the tissues prevented the bad effects of drying and shriveling as wound reactions. On this account, most of the results are based on observations made from pod material.

Killing of material was begun at the end of the first hour following a series of inoculations, and a pod and a leaflet were then removed hourly. Cubes or squares of tissue were cut out at the points of inoculation so as to include the needle path and the limits of infection in so far as this could be ascertained. In the case of atomized leaves picked before infections were visible it was necessary to cut the whole leaf blade into squares and depend upon chance that infections were present. The hourly killing was continued for 50 hours and then daily killing thereafter for a week or more.

Paraffine blocks, when prepared, were sectioned 7 to 9 microns in thickness. Cutting in the plane of the epidermis or at right angles to the path of the needle was found to be as useful as any in tracing the course of infection. Staining was done by Fleming's triple method. The periods in saffranin were as a rule longer than a half hour and in gentian violet from 2 to 5 minutes.

Results

Gross aspects of infection.—In the case of pods attached to the plants, ordinarily invasion was discernible 18 to 20 hours following inoculation, but if the tissue about the needle punctures was cut out and held to the light, invasion was apparent as early as 12 hours by means of a more or less translucent region surrounding the puncture. A computation of the approximate average rate of advancement through the cortical tissue of the pod gave 65 microns per hour. Rapidity of invasion appeared to be influenced most by temperature, as there was a marked increase in the rate at 85° F. or higher as compared with 65° to 70° F. This difference was evident by the earlier appearance of external signs of invasion such as the fading of the normal green color of the leaf and the water-soaked halo on the pods.

Tissues Invaded.—In the wall of the pods, the bacteria in nearly all cases advanced farthest from the needle puncture in the cortical tissue. Within the limits of the cortical region, the greatest progress was made in the portion near the epidermis, although not infrequently it was in a midway zone where the vascular bundles lie and where also there was evidence of considerable storage of reserve food. No special disposition to follow the vascular tissue has been observed that compares with that reported by Gardner and Kendrick (3) in cowpea seedlings. In some instances in the lima bean, however, a series of lengthened parenchyma cells paralleling the tracheae, may be wedged apart or filled with bacteria for some distance along a bundle, but it was the exception for the organisms to be seen within the tracheae, although access to them seemed possible when the needle pierced a bundle. It is thus shown that when discoloration is observed along the veins the bacteria have followed these lengthened parenchyma cells rather than the tracheae.

Invasion of the succulent pith layer lying next to the seeds for any considerable distance was unusual, although one instance was found in which the space advanced had been two mm. in four days and was nearly equal to that in the cortical region. It is possible that the inoculum was placed less advantageously in this deeper layer of tissue.

Lying between the cortex and the succulent pith in the pod wall is a supporting layer consisting of elongated, close-fitting cells that have limited secondary thickening of the walls. In this tissue the parasite made almost no progress, with the exception that the cells broken or pierced by the needle were entered and then progress continued throughout their length.

Such observations as were made of infected leaves in the present work showed that invasion was most rapid within the spongy tissue. Data strongly indicating stomatal entrance of the parasites were obtained, but these have not been considered conclusive enough to discuss at length in this bulletin.

Formation of Zoogloea.—In tracing the course of invasion in detail, the first activity apparent on the part of the bacteria introduced by the needle is the organization, apparently through the normal process of fission and subsequent association, of small masses or colonies (zoogloea) along the line of puncture. A condition eight hours after inoculation is illustrated in Plate I, Fig. a. By differences in staining qualities as well as by the manner of grouping, it is possible to distinguish the masses that have arisen through recent growth from the groups of bacteria that were deposited by the needle but have remained inert. The newly developed masses lie either within or between the lacerated cells. When staining has been properly done, these masses that have arisen through recent growth are revealed as rather closely grouped individuals lying within a very faintly blue-stained or sometimes virtually hyaline matrix or ground material. In many cases this matrix is difficult to distinguish and a differently staining background, such as a cell wall or some effusing cell product, aids greatly in bringing it into relief. In Plate I, Fig. c, the more deeply stained cell wall is in contrast with the hyaline matrix of the bacterial mass. As far as may be told in some instances, similar hyaline ground material may comprise the halo to be seen about individual bacteria lying apart from others. However, the matrix in material a week or more old was found to stain a deeper blue and to exhibit a granular character, (Plate I, Fig. j). It then became evident that each bacterium is surrounded by a clear halo comparing in shape to the bacterium itself. Wherever observed within the host, the masses of bacteria with the accompanying matrix appear as definitely limited entities set off by a distinct margin from any host part or product. For this reason the application of the term zoogloea seems appropriate.

Migration in the Tissues.—As a second step of invasion, the bacterial masses or zoogloea were observed to have advanced along the intercellular spaces of the uninjured cells. In Plate I, Fig. b, a zoogloeal mass is seen advancing into a large intercellular space which thus far is but partially occupied. From what may be observed near the limits of infection, growth of the zoogloea tends to conform to or follow pre-existing passages. For a distance behind the limits of progress, side pressure against the cell walls is not apparent and some intercellular spaces may be but partially or not at all occupied. As the bacteria multiply, however, evidence of considerable pressure against the walls is observed, (Plate I, Fig. d). The pressure is not evenly exerted, as series of bulgings give proof. Cells are wedged apart and some become completely surrounded. Schizogenous cavities are formed and these may become continuous between a series of cells. The mechanical factor in the separation of the

cells is much in evidence. Upon the role or the importance of enzyme action in this connection, the methods of the present study gave no positive data.

In the intercellular spaces, the tips of the zoogloeal masses are characteristically convex in the region near the margin of invasion or in the material fixed 24 hours or so following inoculation. There does not appear to be any close adhesive contact between the substance of the matrix and the cell walls. Often, at the advancing margins, the matrix material is in relatively great amount and the bacteria are relatively few and more widely separated than elsewhere, (Plate I, Fig. c).

Reaction of the Host Cells.—At the limits of invasion, the host cells commonly show no apparent injury or reaction to parasitism. Zoogloea may touch all sides of a cell with no observable result. Somewhat back of the limits of invasion, usually the width of one or two cells, the first effects of parasitism are to be seen. This first effect is plasmolysis, or at least a reaction which resembles plasmolysis. In Plate I, Fig. b, a slight retreat of the plasma membrane is observable in the upper portion of the illustration, while in Fig. d, the phenomenon is far advanced with the protoplasts shrunken to less than half their original proportions. The retreat of the plasma membrane may begin only on one side of a cell where the zoogloea first made contact, or it may start upon the entire periphery. Some care was necessary to be certain that the effect was not due merely to faulty fixation and so study was confined to material in which uninvaded tissues showed the plasma membranes still in contact with the cell walls.

The plasmolytic effect may progress far before any further changes in the protoplasts are discernible. Subsequent changes consist of disintegration in the plastids and along the plasma membrane. The nucleus appears to be the last part of the protoplast to exhibit an effect. It is possible for the various portions of the protoplast to undergo more or less disintegration without the bacteria gaining entrance to the lumen (Plate I, Fig. d). Such digestion with the cell wall intervening would indicate the diffusion of bacterial enzymes through the wall. This phenomenon is confined to the region considerably behind the margin of invasion, and is especially to be observed where there is a great accumulation of the parasites between the cells.

While it appears to be commonplace that the observable reactions to parasitism occur back of the margin of invasion, some instances of the opposite condition are to be found. At those places where active advancement between the cells has stopped or has been checked, possibly by meeting considerably smaller intercellular spaces, a great development of bacteria immediately near the margin of invasion appears to exert an effect, probably enzymatic, out in the uninvaded tissue for a distance of one or two cells which then exhibit the plasmolytic reaction.

Entrance to Cells and Intracellular Development.—While the migration of the bacteria through a tissue is mostly an intercellular phenomenon, entrance is ultimately gained to the lumina of a considerable

number of cells, especially after the accumulation of the organisms between the cells has become great. The actual manner of entrance is difficult to ascertain, although both mechanical pressure and the disintegration of the cell walls in certain places appear to play a role. Wherever a cell is entered, the intercellular space opposite is gorged with bacteria (Plate I, Fig. e).

In practically all cases, the plasma membrane has withdrawn a considerable distance before an entrance is made through a cell wall (Plate I, Fig. e). In the space left between the wall and the shrunken protoplast, growth of the bacteria does not appear to occur. Pressure from the intercellular space apparently crowds the intruding portion of the zoogloea over against the shrunken protoplast and the subsequent intracellular development is mostly an extension of the zoogloea by growth, which is not only about but within the limits of the protoplast (Plate I, Fig. f). The multiplication of the bacteria can be followed through a number of stages in which the protoplasts gradually become engulfed and the cell lumina completely packed with the organisms, as illustrated in (Plate I, Fig. d). Remnants of the plastids or nuclei often may be seen in the filled cells.

During some stages within certain cells, the zoogloea in its intracellular development exhibits peculiar lobular outgrowths and sinuous depressions and recesses. The general aspect of these features may be described as cerebroid (Plate I, Fig. g). They were noted especially where the bacteria entered broken ends of the rather long cells of the supporting tissue. Ultimately this peculiar form of zoogloeal growth seems to be lost by crowding and only a common mass of bacteria is to be seen. Some cells become densely filled except for a narrow margin next to the wall, and this may be in part the space produced by the retreat of the plasma membrane.

Effects on Cell Walls and Lysigenous Cavities.—In general, cell walls appear to undergo no great change through the presence of the bacteria, with the exception, of course, that separation along the line of the middle lamella occurs. Aside from the openings through which the parasites enter the lumina, the contact of extensive masses of bacteria produce little discernible change either in thickness or texture of the walls, at least little that the cytological methods employed have brought to light. The apertures permitting entrance to the lumina are usually relatively small, and in some instances there is no evidence as to the place the organisms entered the cells. Although in Plate I, Fig. h, one small and two large apertures are illustrated, most of the walls are still apparently intact. Lysigenous cavities are thus few and not extensive, and the condition illustrated in Fig. h is an uncommon advance in tissue disintegration. In this connection, an observation worthy of mention is that most of the cells, in which the parasites entered the lumina, had thin walls.

Change in the Form of the Bacteria.—An important point which indicates that *Bacterium vignae* may have a life cycle comparable to that

described by Nixon (6) for *Bacillus amylovorus* is in a change of size and form of the rods. This is observable when bacteria of recent infections and plainly in the active process of fission are compared with those in older disease spots where fission has ceased and a state of inactivity has ensued. Representative of the first condition, which may be regarded as vegetative, were bacteria in material 8 to 25 hours old, while representative of the latter, which may be regarded as dormant, were bacteria in pod spots 7 to 10 days old. Although in the latter case the spots had several days past ceased to enlarge and were depressed, the parasitized tissues still possessed a considerable degree of succulence. The mode for length in the vegetative state was found to be 1.7 microns and the range .9 to 2.2 microns, while in the reduced or dormant form, the mode for length was .8 microns with a range of .5 to 1 micron. The contrast in size and form is illustrated in Plate I, Figs. i and j. The reduced forms stain more densely than the actively dividing rods. Frequent among the reduced forms of the bacteria is the arrangement in pairs of two very short rods or sometimes spheroid bodies which probably represent a last fission of the actively dividing stage.

Discussion

In some important respects, the behavior of *Bacterium vignae* is similar to *Bacillus amylovorus* as reported by Nixon (6). He reports that "*Bacillus amylovorus* migrates through apple tissue in the form of a zoogloea; in the early stages, the most extensive migration is intercellular; due to the action of the organism, two distinct types of ultimate injury result, viz, the formation of schizogenous and lysigenous cavities." He reports further that "In the life of *B. amylovorus* there are two phases or periods: a vegetative stage and a pseudofructification. The major portion of the intercellular migration in the form of zoogloea comprises the vegetative phase. Intracellular migration with, ultimately, the formation of cysts, characterizes the pseudofructification phase." These aspects of *Bacterium vignae* are essentially the same.

It is only in less important respects that differences between the two parasites are to be seen. Nixon (6) shows the matrix material of *Bacillus amylovorus* to be relatively great in amount as compared with the number of bacteria and also more deeply staining than is the case with *Bacterium vignae*. In the vegetative stage of the latter organism, it has been necessary to illustrate the presence of the matrix by contrast with the cell walls and the bacteria, while with *Bacillus amylovorus* it appears at all stages as a blue-staining granular substance. A possible explanation of this difference is that *Bacillus amylovorus* is gram-positive while *Bacterium vignae* is gram-negative.

Regarding the consistency of the matrix, Nixon (6) states that in *Bacillus amylovorus* it appears to be more than a "watery liquid," as Miss Bachman (1) thinks, and that the bacteria appear to be embedded in a jelly-like substance. In *Bacterium vignae*, features that give some indication of the consistency of the matrix are the convex margins pre-

sented by the zoogloea in the younger stages and the lack of close adhesive contact with the cell walls. These features would appear to indicate a consistency greater than water.

There is obviously a great contrast between the two organisms in the rate and distance they migrate through their host tissues. Comparison of reported data indicates that *Bacillus amylovorus* possibly migrates 40 times more rapidly. However, it appears that *Bacterium vignae*, which causes but small lesions, reaches the limits of its progress in a comparatively brief time. Progress in spots several days old was but slightly wider than in some fixed 27 to 42 hours after inoculation. Moreover, observations of the material indicates that probably a third of these brief periods elapse before infections gain momentum. Except for Fig. h of the lysigenous cavities and Fig. j of the reduced form of the bacteria, all the stages illustrated in Plate I are to be found in material killed less than 42 hours after inoculation. What checks further progress in a spot-forming organism would be most interesting to ascertain.

The development of the dense masses of *Bacterium vignae* within the cells of lima bean seems to parallel only the early stages in the formation of the cysts which Nixon (6) describes for *Bacillus amylovorus* in apple twig tissue. There the bacteria become so closely aggregated that the individuals are no longer distinguishable. In no case during the examination of invaded cells in lima bean has it been impossible to discern the bacterial character of the dense bodies, at least up to 10 days following inoculation, which has been the greatest age of any material prepared. No striking difference was to be found in the general aspect of the masses when the 10-day preparations were compared with some that were but 27 hours old, other than the reduction in the size of the bacterial cells and the deeper staining character of these as well as the matrix in which they were embedded. In view of the greater density with which the matrix of *Bacillus amylovorus* stains in comparison with that of *Bacterium vignae*, it appears possible that this may be a factor in obscuring the bacterial cells of the former organism during its encysted condition.

In the case of *Bacterium vignae*, there was no indication that the dense masses contracted any measurable degree with age. It may be that the bacteria packed within the host cells are better able to resist drying or other adverse environmental conditions, but these masses as thus far observed do not appear to justify the term cyst as used by Nixon (6). It is possible, however, that material exhibiting features more indicative of an adaptation for dormancy might be found in older or dry tissue. *Bacterium vignae* has been shown to retain viability for a long time in herbarium material. Tisdale and Williamson (9) report that a pathogenic strain was isolated from leaves preserved for two years.

The period when the rods of *Bacterium vignae* undergo their transformation of size and form has not been ascertained definitely. The vegetative stage, in which the bacterial cells show the most typical and distended rod form as well as the greatest degree of uniformity in the staining of their general structure, has short duration. Rods of this

type occur only near the margin of invasion or about protoplasts becoming engulfed; that is to say, only in the earliest periods of zoogloeal development. As soon as intercellular spaces or cell lumina become crowded with bacteria, an increase of granulation is to be observed in the internal structure of the rods. Reduction in size seems to be under way also, but this is scarcely measurable. Further change into the much reduced, densely staining condition like that in Plate I, Fig j, has not been followed.

While taking account of the parasitism of a spot-forming parasite like *Bacterium vignae*, it seems pertinent to consider the activities reported for some other bacterial types. Geitler (2) describes the entrance of *Polyangium parasiticum*, a species of myxobacteriaceae, into the cells of the alga Cladophora. He states that the bacterial mass attacks the outside membrane, separates it into layers, and by successive dissolution of the layers produces an opening large enough for an entrance. He states that the first reaction of the protoplast is a slight plasmolysis which follows rapidly along all sides. In digesting the host cell, plasma, nucleus, chromatophores, pyrenoids, and starch are attacked in like measure. As a rule the wall membranes resist attack, although these are occasionally dissolved.

Hill, a contemporary worker whose paper has been published in abstract (4), found in studying the migration of *Bacterium tumefaciens* in the pith tissue of tomato that the parasites organize themselves into the type of zoogloea described in this bulletin.

Robinson and Walkden (8) apply the term zoogloea to the intercellular masses of *Bacterium tumefaciens*, though the present conception of migration was not described.

The behavior of the thread-like zoogloea or infecting strands which Pierce (7) found in the root-tubercles of burr clover appears to have a degree of similarity with the peculiar formation of the zoogloea of *Bacterium vignae* within some invaded cells of lima bean. Pierce states that the infecting strands of bacteria grow definitely, chemo-tropically, through the cells and also toward the nuclei which they penetrate. Pierce's illustrations show some lobular branching of the infecting strands.

The factors which dispose a parasite largely to certain tissues invite investigation. In lima bean, *Bacterium vignae* showed a marked preference for the chlorophyll-bearing cortex of the pods and the spongy tissue of the leaves. These tissues are characterized by wide intercellular spaces, a large supply of oxygen, and usually a plentiful supply of immediately available food. The bacteria placed in the succulent pith and the supporting tissue of the pod wall multiplied and filled torn cells but made little progress otherwise. The narrowness of the intercellular spaces would appear to be a great obstacle to invasion, and lack of available food and oxygen may be contributing obstacles.

It is necessary, in discussing the final effects of parasitism such as that caused by *Bacterium vignae*, to make a distinction between the effects involving the cell walls or the protoplasts and the general collapse of the

tissues. This collapse of the tissues in which cells flatten upon one another and the lesions become depressed seems to be caused mostly by loss of turgor and drying out, although it is probable that disintegration of the cell walls is a contributing factor.

SUMMARY

The migration of *Bacterium vignae* through the tissue of lima bean is in the form of zoogloea. The matrix material in which the bacteria are embedded stains faintly in early stages but more deeply in later stages. Progress through a tissue is mostly intercellular.

The first reaction of the host cells resembles plasmolysis. This is followed by the disintegration of the plasma, the plastids, and the nuclei. It is possible for these effects to appear without entrance of the bacteria to the lumina.

The lumina of a considerable number of cells are invaded ultimately, but the apertures of entrance are usually small and sometimes difficult to locate. Bacterial growth within and about the shrunken protoplasts occurs, engulfing these and finally filling the lumina completely. The dense masses of bacteria occupying the lumina do not, however, assume the characteristics of cysts.

Within a week to 10 days following inoculation, bacterial rods were found to have undergone a change of size, form, and staining reaction.

The activity of the parasite causes the following types of final injury: schizogenous cavities which are common and extensive, lysigenous cavities which are usually limited in number and extent, and the general collapse of the tissue.

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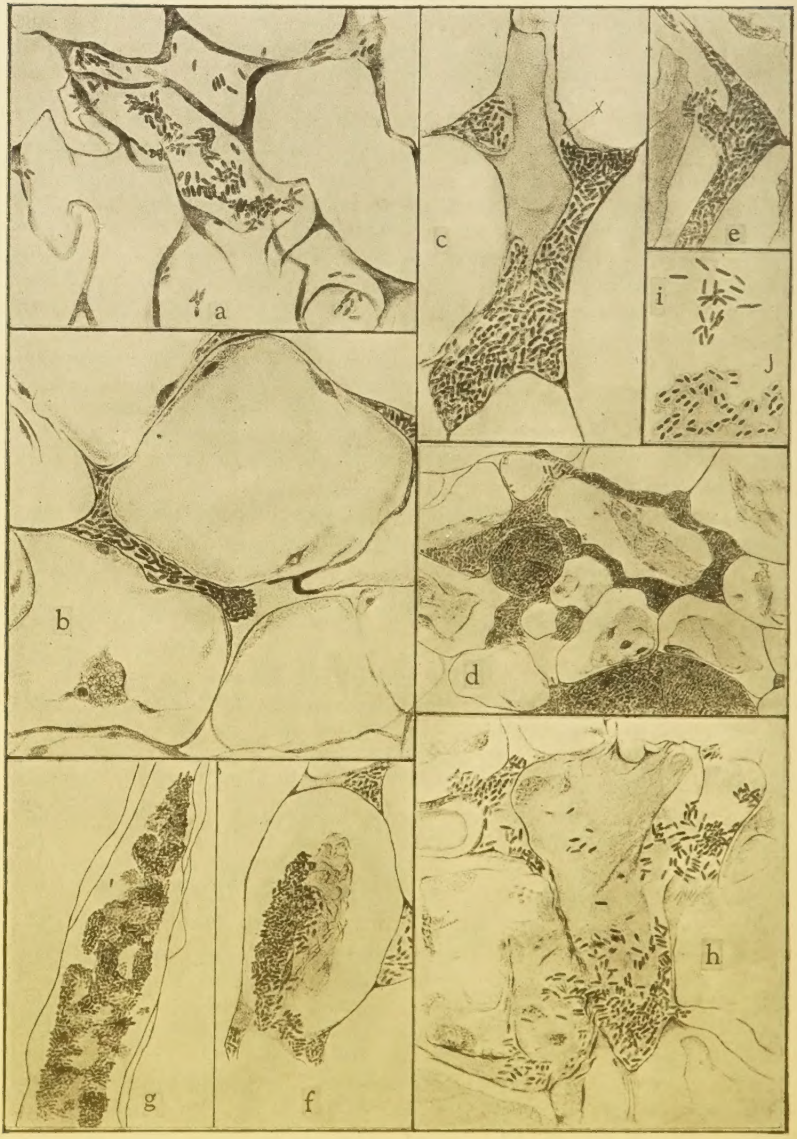
a
b
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Explanation of Plate I

Fig. a.—The central group of bacteria comprises a beginning stage in the organization of a zoogloea within torn cortical cells along the needle wound. Pod tissue fixed eight hours after inoculation. x 2000.

Fig. b.—A zoogloeal mass advancing into an intercellular space at the margin of invasion. x 2000.

Fig. c.—Zoogloeal masses or branches within intercellular spaces of cortical tissue near the margin of invasion. The faintly stained or virtually hyaline matrix is contrasted with the cell walls and the bacteria. Its characteristic convex margin in advance of the bacteria is indicated by x. Pod material fixed 27 hours after inoculation. x 2000.

Fig. d.—The condition of invaded cortical tissue 42 hours after inoculation, illustrating degrees of disintegration of the protoplasts, a schizogenous cavity and cells packed with bacteria. x 1000.

Fig. e.—An early stage in the invasion of a cell lumen. x 2000.

Fig. f.—An intermediate stage in the intracellular growth of the zoogloea showing a shrunken protoplast being engulfed by bacteria. Pod material fixed 27 hours after inoculation. x 1750.

Fig. g.—A peculiar cerebroid form of zoogloeal development within a lengthened cell of the pod supporting tissue, 25 hours after inoculation. x 1750.

Fig. h.—The formation of a lysigenous cavity within the cortical tissue of the pod four days after inoculation. x 2000.

Figs. i-j.—Bacteria in the vegetative stage contrasted in size, form, and staining reaction with bacteria in an inactive or dormant stage found in material fixed 10 days after inoculation. x 2000.